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Functional characterization of toposomes from sea urchin blastula embryos by a morphogenetic cell aggregation assay

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This paper documents the evidence that the large oligomeric glycoprotein complexes of unknown function first isolated as 22S particles from sea urchin embryos are the sole agents responsible for the adhesive integrity of sea urchin blastula embryos. The conclusion rests on the demonstration that polyclonal IgG (as serum or monovalent Fab) against whole membranes or butanol-solubilized components of membranes, as well as against the purified particle itself, completely blocks reaggregation of dissociated blastula cells and that this inhibition is reversed by neutralization of the inhibitory antibodies with purified 22S antigen. An essential aspect of the evidence is the combination of quantitative endpoint titrations in microtiter wells with the qualitative parameters of morphogenesis. The new data complement previous evidence that morphogenesis is mediated by a general class of particles, toposomes, responsible for mechanical linkage between cells and their positional guidance in embryogenesis.

Key words: toposomes/cell adhesion/morphogenesis/cell contact blocking antibodies/glycoprotein

Introduction

It has often been assumed that cell adhesion molecules are involved not only in mechanical linkage between cells, but in positional guidance as well. A search for molecules with such a dual function requires an assay and a suitable experimental system. Sea urchin embryos appeared ideal for a morphogenetic cell adhesion assay because they can be dissociated into single cells which under proper conditions reaggregate to form developing embryos (Guidice, 1962), and thus offer a way of assessing both quantitative aspects of cell adhesion and the qualitative features of differentiation and morphogenesis (Noll *et al.*, 1979, 1981). Using such assays as a guide for the purification of active molecules, Noll *et al.* (1985) isolated large oligomeric glycoprotein complexes which they called toposomes for their ability to mediate cell adhesion and to express positional information.

Here we describe the assay and document the evidence that the 22S glycoprotein complex described previously (Malkin *et al.*, 1965; Kane, 1965; Stephens, 1967; Borisy and Taylor, 1967; Infante and Nemer, 1968; Kondo, 1972; Kondo and Koshihara, 1972; Il *et al.*, 1978; Harrington and Easton, 1982; Kari and Rottmann, 1985) is the sole component responsible for the adhesive integrity of the sea urchin blastula.

In the first part we document the validity of the assay and in the second we show that highly purified 22S particles neutralize all reaggregation inhibiting antibodies, regardless of whether they have been raised against epitopes associated with purified membranes or the subset solubilized with butanol from purified membranes or non-cytolytically from the surface of live cells.

Results

Cell density

The cell density is fairly critical for reaggregation, as is evident from Figure 1 which shows the reaggregation patterns obtained from *Paracentrotus lividus* cells after 5 h with an input corresponding to an 8-fold range from 2.5×10^4 to 2×10^5 cells per well (Figure 1, a–d). The rate of reaggregation, as reflected in the number and size of aggregates, increases with the number of cells added. At 10^5 cells per well, the aggregates are about the size of normal blastulae (c). At lower inputs they are smaller (a,b), at higher inputs they are larger and tend to coalesce into sheets that cover the bottom of the well (d). If the cell density is adjusted to 10^5 per well initially, it is not necessary to dilute the cells to break up large aggregates as recommended in our first paper (Noll *et al.*, 1979).

Cell density also determines the developmental fate of an aggregate because reaggregation is followed by the formation of a smooth ciliated epithelium, which, once formed, prevents further addition of cells. Hence, at low density, aggregates will detach from the surface and swim around before reaching the critical size that seems to be required for gastrulation and subsequent stages of development. Thus, below an input of 4×10^4 cells per well the potential of development beyond the blastula stage is greatly reduced.

Effect of homospecific and heterospecific antiserum

The different effects produced over a 4-fold range of cell densities by heterologous and homologous antiserum are illustrated in rows e–g and i–k in Figure 1. After 5 h, reaggregation of *P. lividus* cells in the presence of anti-*Arbacia* serum was normal or even somewhat enhanced (e–g), while it was completely inhibited in the presence of homospecific antiserum (i–k). Closer inspection reveals that unlike homospecific Fabs which prevent cell agglutination and aggregation (Figure 2p), homologous serum produces agglutination: the cells form a characteristic lattice within which the rounded contours of individual cells are still visible (Figure 1, i–k). The appearance of this pattern does not change until many hours later when the cells begin to lyse. At that time (100 h), some of the controls had developed to the pluteus stage, as exemplified by the giant pluteus shown in Figure 1h. The agglutination patterns produced in the presence of homologous antiserum is characteristic of genus-specific antibodies rather than some inhibitory serum factor because it disappeared when the IgG fraction was removed from the serum (Figure 1l). IgG-depleted serum clearly stimulates reaggregation as evident from the larger average aggregate size as compared with the control (compare l with b in Figure 1). Similarly, heterologous serum consistently stimulated reaggregation as apparent from comparing the average aggregate size in Figure 1, e–g with the controls in a–c. The effect is most pronounced at 5×10^4 cells per well (Figure 1f) in which some

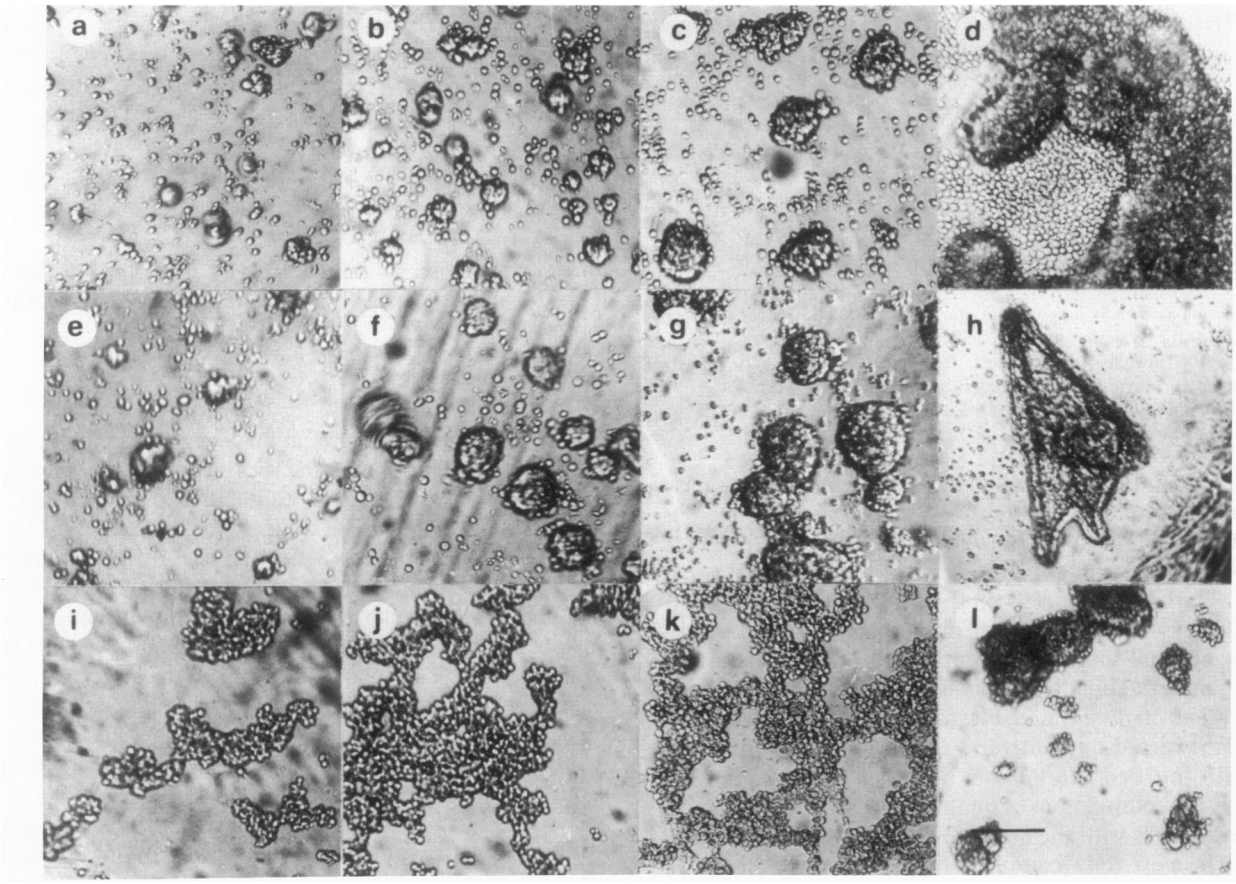

Fig. 1. Effect on reaggregation of increasing cell density in the presence and absence of antibodies. The first well in each row contained 2.5×10^4 *P. lividus* cells **(a, e, i)**; the second 5×10^4 **(b, f, j)**, the third 10^5 **(c, g, k)**. The fourth **(d)** contained 2×10^5; **(h)** 7×10^4, and **(l)** 4×10^4. No antibodies in **a–d**; 1.25 μl of anti-*Arbacia* immune serum in **e–g**, or of anti-*Paracentrotus* serum **(i–k)**. All photographs were taken after 5 h, except **(h)** which shows a no-serum control after 100 h. In **(l)** reaggregation is shown in the presence of 32 μl of IgG-depleted anti-*Paracentrotus* serum 1223B. Magnification: the horizontal bar corresponds to 100 μm in all figures.

of the aggregates are already swimming, as indicated by the blurred appearance in the photograph.

Heterospecific serum initially causes agglutination as well, because the sera employed here contain cross-reacting bivalent antibodies directed against surface components shared by *Arbacia lixula* and *P. lividus* as revealed by Ouchterlony analysis (Noll *et al.*, 1981). However, this agglutination is transient and gives way to the normal reaggregation patterns seen in Figure 1 e–g. Hence the genus-specific agglutination patterns produced by homologous serum can be used diagnostically as well as the corresponding Fabs. With the homologous serum used for the experiment in Figure 1, the minimum effective concentration was 1.25 μl/100 μl culture at the lower cell input (i, j), 2.5 μl/100 μl at the higher cell density (k). Such a concentration dependence would be expected if most of the effective antibodies were bound.

Comparison of serum with IgG and Fab

In Figure 2, the effects of homospecific and heterospecific sera are compared with the corresponding parent IgGs and Fabs. The results show that, as expected, the action of IgG parallels that of serum in that it causes the same type of reticular agglutination in the homospecific cells while having no effect on the heterospecific reaggregation.

In the first two rows of Figure 2, the effects of increasing concentrations ranging from 32 to 256 μg/0.1 ml of heterospecific (a–d) and homospecific (e–h) IgG are compared 4 h after the start of reaggregation. Even at the highest concentration (d), IgG from rabbits immunized with butanol extract from *Arbacia* membranes had no inhibitory effect, and the extent of reaggregation is indistinguishable from the control (l). The early stages in the formation of an epithelial sheet are recognizable. By contrast, homologous IgG completely inhibits reaggregation at the lowest concentration tested (32 μg/0.1 ml) (Figure 2e). The lowest inhibitory concentration of the corresponding antiserum was 0.5 μl (Figure 3c). This corresponds to ~5 μg IgG/0.1 ml of reaggregation medium. It follows that IgG accounts for all the inhibitory activity in serum in agreement with the finding that IgG-depleted serum fails to inhibit reaggregation and often stimulates (Figure 1l). The inhibition of reaggregation by 32 μg/0.1 ml of homologous IgG seen after 4 h (Figure 2e) still persists after 25 h (Figure 2i). By contrast, 272 μg/0.1 ml of heterologous IgG (Figure 2j) did not prevent development of the reaggregates to blastula-like structures that resemble closely the controls without IgG (Figure 2k). For comparison, the last row shows the inhibition patterns produced when a preparation of homologous Fab is added at increasing concentrations to reaggregating cells (Figure 2m–o). Unlike serum and IgG, Fab at inhibitory concentrations shows a carpet of evenly distributed single cells which begin to form clusters that increase in size, number and contour sharpness as the Fab is diluted out. Some residual clumping seen after 7 h (Figure 2o) has disappeared by 24 h (Figure 2p) because at this relatively low Fab concentration, dissociation of the clumps formed before addition of the Fab is slow.

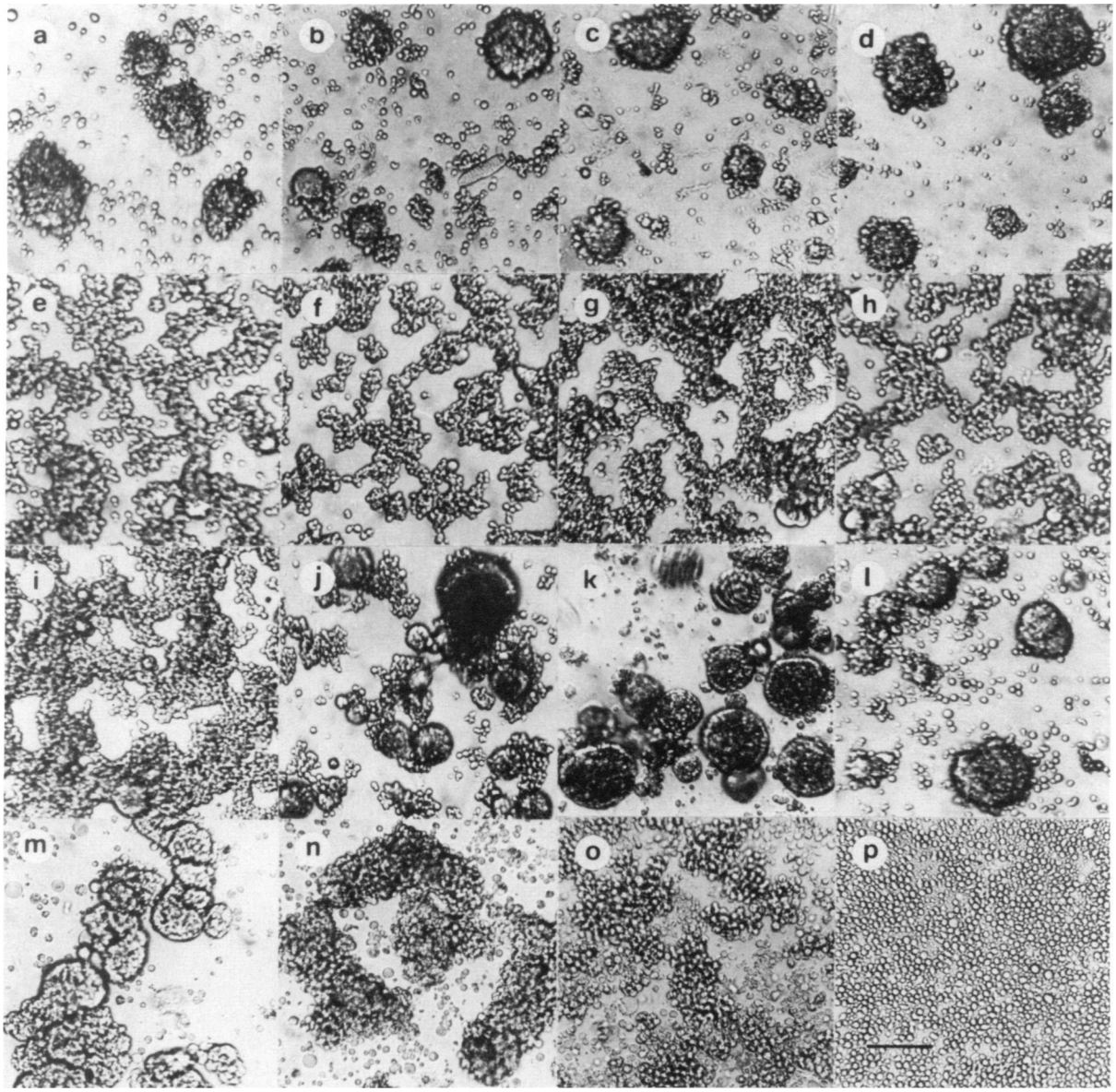

Fig. 2. Effect on reaggregation of various concentrations of homospecific and heterospecific IgG. The IgG was from anti-*Arbacia* immune serum **(a–d, j)** or anti-*Paracentrotus* immune serum **(e–i)**. The first horizontal row contained the following amounts of IgG in μg: 34 **(a)**, 68 **(b)**, 136 **(c)**, and 272 **(d)**; the second row 32 **(e)**, 64 **(f)**, 128 **(g)** and 256 **(h)**; the third row 32 **(i)**, 272 **(j)**, 0 **(k)**, 0 **(l)**. The fourth row contained the following amounts of anti-*Paracentrotus* Fab in μg: 4.5 **(m)**, 18 **(n)**, 71 **(o)**, 53 **(p)**. All wells received 10^5 *P. lividus* blastula cells in a total volume of 0.1 ml. The photographs were taken after the following reaggregation periods: 4 h **(a–h, l)**, 25 h **(i–k, p)**, 7 h **(m–o)**.

Neutralization of reaggregation blocking antibodies with butanol extract or purified 22S complex

A typical endpoint titration by 2-fold serial dilution with homologous antiserum is shown in the first row (a–d) of Figure 3. The readings were taken 3 h after the start of reaggregation. At the lowest concentration (0.25 μl/0.1 ml; Figure 3d), the reaggregation pattern was indistinguishable from the control (Figure 2l). The endpoint (Figure 3c) is characterized by elongated aggregates lacking well-defined epithelial borders that are intermediate between the lattice pattern typical of complete inhibition and the aggregates with partial epithelium corresponding to the controls (Figure 3d and Figure 2l). Thus, the endpoint is unambiguously identified by that microtiter well that contains 0.5 μl of antiserum per 0.1 ml well (Figure 3c).

To assay the contact protein activity, we added increasing amounts of butanol extract to a fixed concentration (either 1.0 or 5.0 μl/0.1 ml) of antiserum. At the lower serum concentration, which corresponds to about twice that of the inhibition endpoint, the lowest amount of butanol extract (1.5 μg protein/0.1 ml) completely neutralized the inhibitory activity of the serum (Figure 3e). At the five times higher concentration of antiserum, reversal is seen at a correspondingly higher concentration of butanol extract (Figure 3k), but the neutralization remains incomplete even at the highest concentration of the extract (Figure 3l). The remaining three rows show the same samples 19 h later (Figure 3 a–d → m–p; e–h → q–t; i–l → u–x). At the lowest concentration of antiserum that completely inhibits after 3 h (Figure 3b), there is extensive recovery after 22 h, which manifests itself by the appearance of blastula-like embryoids with clearly defined epithelial sheets (Figure 3n). Recovery appears as a process of 'nucleation' at the points of highest cell density within the reticular patches. At still lower

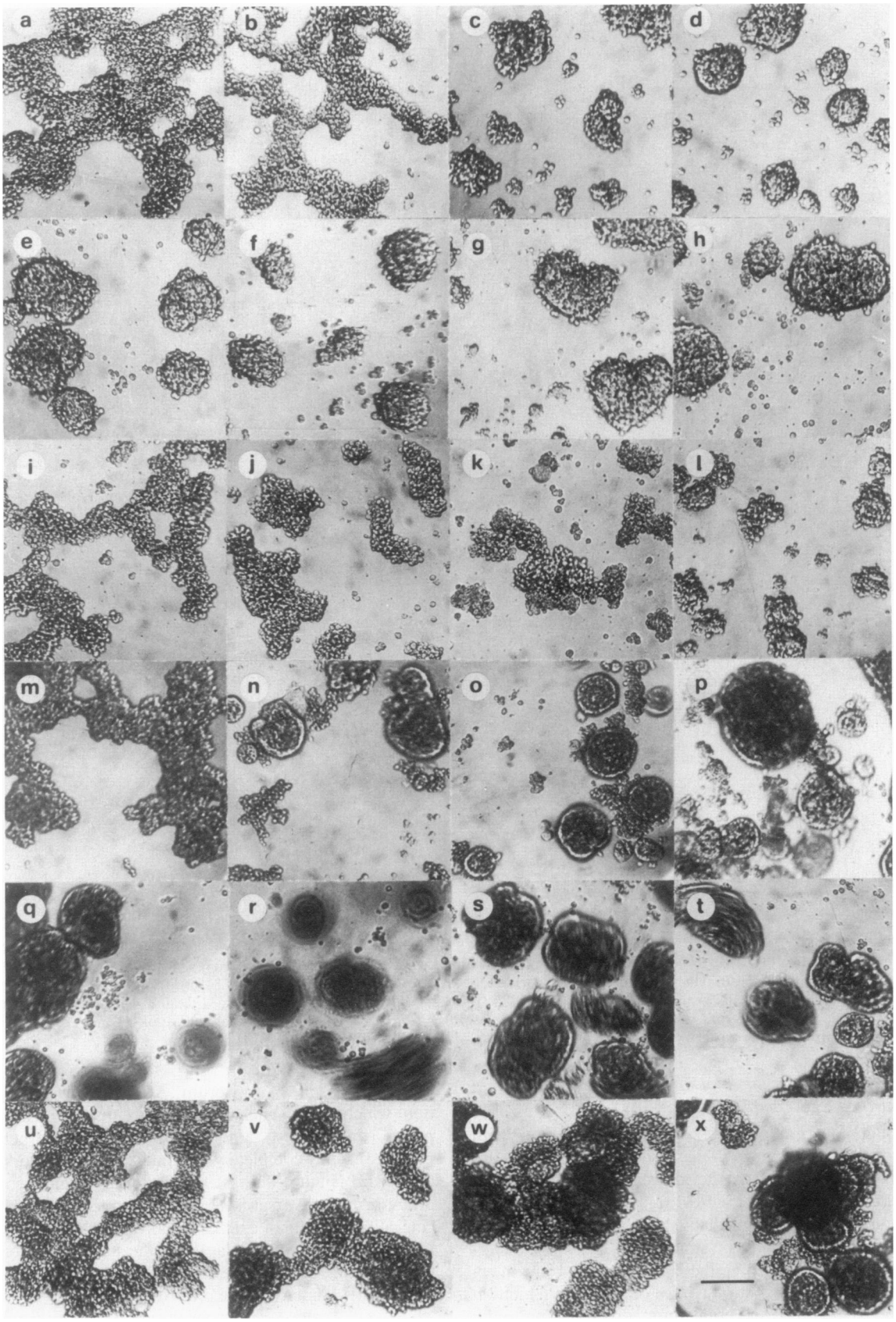

Fig. 3. Endpoint titration of inhibition by IgG and of reversal by immunoadsorption with butanol extract. The first horizontal row illustrates the inhibition endpoint as homologous anti-*Paracentrotus* serum is diluted out. Additions were in μl: 2.5 **(a)**, 1.0 **(b)**, 0.5 **(c)**, 0.25 **(d)**. The second row shows reversal of inhibition at a constant concentration of 1.0 μl serum/0.1 ml and the following amounts of butanol extract from membranes in μg: 0.8 **(e)**, 1.6 **(f)**, 3.2 **(g)**, 6.4 **(h)**. The third row shows partial reversal at a constant concentration of 5.0 μl serum and the same quantities of butanol extract in **i**–**l** as in **e**–**h**. Rows 1–3 show the state of reaggregation after 3 h; rows 4–6 show the same wells after 22 h.

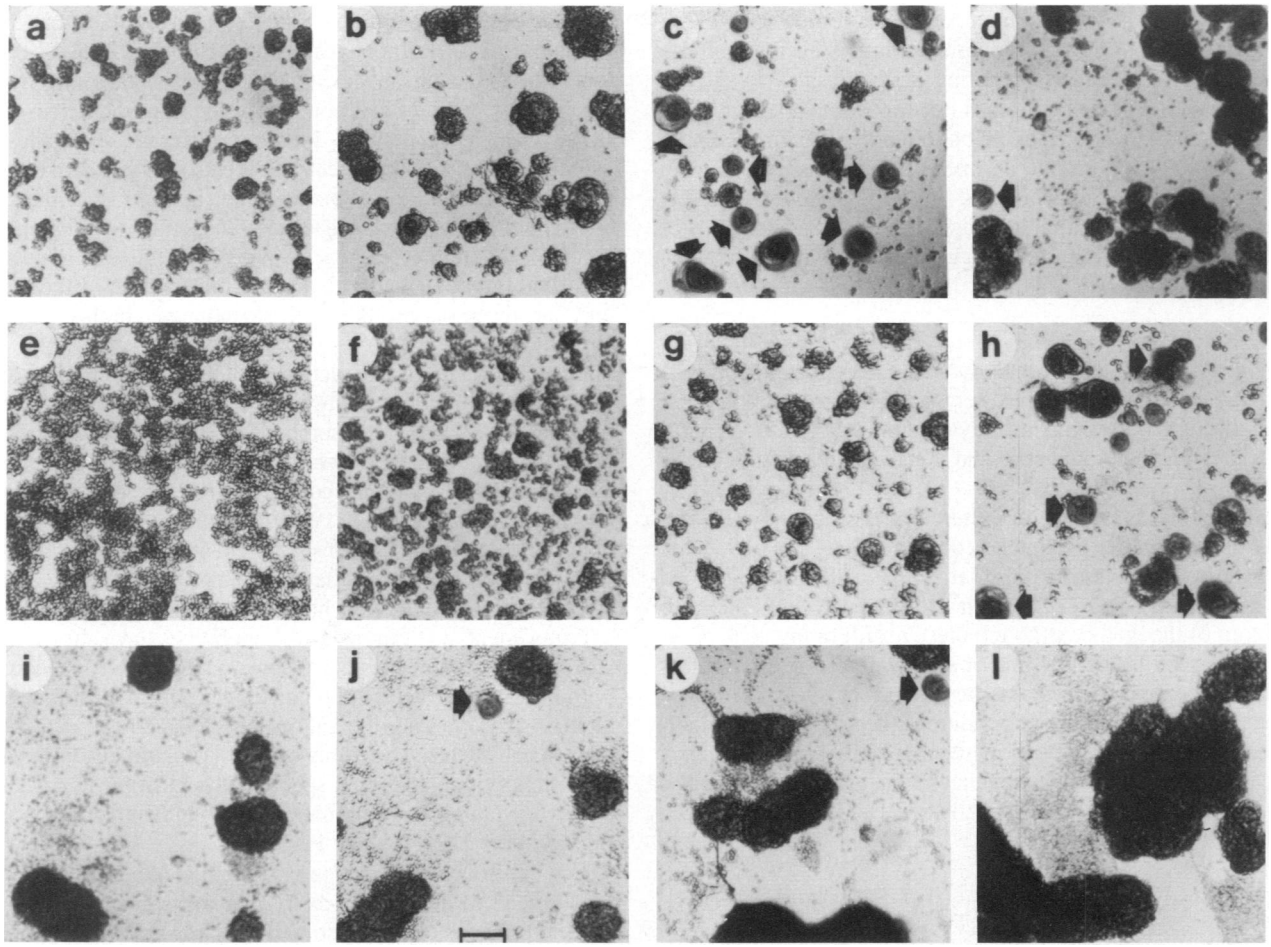

Fig. 4. Inhibition of reaggregation by IgG against 22S complex and reversal by 22S antigen. Increasing concentrations of highly purified 22S complex (**b – d** and **f – h**) were titrated against two inhibitory levels of IgG (34 μg per well in **a – d**; and 85 μg per well in **e – h**. The wells contained the following amounts of 22S complex in μg: 0.05, 0.1, 0.25 (not shown), 0.5 (**b,f**), 1.0 (**c**), 2.5 (**g**), 5.0 (**d,h**). Controls without IgG and 22S complex (**i,j**). Inhibition of reaggregation with IgG alone: 34 μg (**a**), 85 μg (**e**). Stimulation of reaggregation by 5 μg of 22S complex alone (**k,l**). Spinning aggregates (arrows) are seen in **c, d, h, j** and **k**. The pictures not shown were similar to (**a**) for 0.05, 0.1 μg, to (**g**) for 0.25, and to (**h**) for 2.5 μg of 22S complex in the presence of 34 μg IgG; to (**e**) for 0.05, 0.1 and 0.25 μg and to (**f**) for 0.5 μg 22S complex in the presence of 85 μg IgG. Photographs were taken 24 h after the start of reaggregation.

serum concentrations, the recovery after 22 h (Figure 3o, p) approaches the picture seen in the serum-free controls (indistinguishable from p). The reversal by butanol extract resulted after 22 h in the formation of rapidly spinning blastulae (Figure 3q – t), dramatically illustrated by the blurred circular motion in the photographs. By contrast, the controls have not yet reached the free swimming state, in agreement with our earlier observations that butanol extracts stimulate reaggregation above that of the controls (Noll *et al.*, 1979, 1981). Reversal at the 5-fold higher antiserum concentration after 22 h (Figure 3u – x) is less complete and corresponds to that seen after 3 h; only at the highest concentration of butanol extract (6.4 μg/0.1 ml) has development reached the blastula stage with sharply defined epithelial sheets (Figure 3x) similar to the control (Figure 2k).

Purified 22S particles were equally active in neutralizing the reaggregation-blocking antibodies. We tested sera and IgG from rabbits immunized with the following preparations from blastula embryos of *Tripneustes gratilla*: (i) purified membranes; (ii) butanol extract from purified membranes; and (iii) purified 22S particles. The inhibitory titer of sera from rabbits immunized with purified 22S particles was the highest ever obtained, ranging from 0.25 to 1.0 μl of undiluted serum, or ~2.5 – 10 μg of purified IgG per 100 μl sea water and 10^5 cells per microtiter well. In all of these cases (i – iii), complete reversal of inhibition was produced by mixing an excess of purified 22S particles with the antibody preparation.

A representative example is illustrated by the 24 h cultures photographed in Figure 4. In this experiment we titrated concentrations of 22S complex increasing over a 100-fold range against 34 μg (Figure 4a) or 85 μg of anti-22S IgG. The endpoint of inhibition of this IgG preparation, defined as the last well in a dilution series showing inhibition of reaggregation as compared with the controls, was determined separately (8.5 μg). At the lower IgG concentration, complete reversal by the 22S complex is seen at 0.5 μg/100 μl (Figure 4b), stimulation over the control as judged by movement (arrow heads) at toposome concentrations ranging from 1 μg (Figure 4c) to 5 μg (Figure 4d). At the higher antibody concentration, correspondingly higher toposome concentrations were required for neutralization (Figure 4g) and stimulation (Figure 4h). The effect of reversal is clearly seen by the extent of epithelium formation at the equivalence point (Figure 4b, g), while stimulation is illustrated dramatically by the fact that the blastula-like embryoids are spinning (Figure 4c, d, h). At a photographic exposure time of 0.25 s, this produces concentric blurs (arrow heads in Figure 4). Complete reversal was also observed when the purified 22S complex was titrated

against IgG raised to purified membanes or to crude butanol extract. It follows that the 22S complex contains all the epitopes recognized by aggregation inhibiting antibodies.

Controls with neither antibodies nor toposomes (i,j) or toposomes alone (k,l) are shown in the bottom row of Figure 4. Spontaneous reaggregation produces somewhat larger aggregates with delayed formation of an epithelial sheet (i,j) except in a few smaller aggregates that are spinning (j). High concentrations of toposomes (k,l) results in giant aggregates and a few smaller, spinning embryos (k).

Serum factors

Homologous antisera that strongly inhibited reaggregation became as stimulatory as heterologous sera after removal or IgG by passage through a protein A affinity column. Maximum stimulation varies with individual sera and is usually reached between 0.5 and 2 μl.

Another effect of serum on cultures of dissociated cells frequently observed after prolonged culturing (24 – 100 h) was the outgrowth of a network of polyfilamentous and spindle-shaped cells (Timourian *et al.*, 1973) that are flattened and adhere to the bottom of the wells. Although these proliferating cells occasionally are seen in the absence of serum, in our present series of >100 culture wells we have only seen them in the presence of serum under non-inhibitory conditions (heterospecific or IgG-depleted). They appear to be of mesenchymal origin because they eventually begin to produce spicules. This effect of serum has been noted before (Okazaki, 1975; Harkey and Whiteley, 1980; Mintz and Lennarz, 1981).

Discussion

In this paper we document the functional identification of a large glycoprotein complex from sea urchin blastula membranes. The function and biological significance of this complex, first isolated from sea urchin cytoplasm by Malkin *et al.* (1965), has been an enigma and the subject of considerable debate. Here we show that this complex binds all reaggregation-inhibiting antibodies directed against the blastula cell surface. Conversely, immunization of rabbits with the purified complex produced sera with a high reaggregation inhibiting titer. It follows that the 22S glycoprotein complex contains all the epitopes of the contact site. Indeed, evidence that the 22S particle is the sole structure containing the contact site comes from reconstitution experiments. Non-cytolytic washing with sea water containing 2.5% butanol removes the complex from the cell surface, and the depleted cells have lost the capacity for reaggregation as well as for movement and attachment to the substratum (Noll *et al.*, 1979). All of these functions are restored by readdition of the glycoprotein particles (Noll *et al.*, 1985).

The experiments reported here and previously (Noll *et al.*, 1979, 1981) show that the contact site is genus specific. The only other feature of the complex known to be genus specific is the SDS gel pattern of fragments generated by proteolytic processing of the particles during development. Hence processing probably involves the contact site. Processing, however, does not result in breakdown of the particle because the fragments are held together within the particle by S-S links and non-covalent bonds (Kari and Rottmann, 1985; Noll *et al.*, 1985).

Fab fragments of the genus-specific antibodies that inhibit reaggregation cause dissociation of live embryos into single cells as well (Noll *et al.*, 1981). The same effect is produced by Con A or chelation of Ca^{2+} without loss of the complex from the cell surface. These results, together with the fact that the 22S particles are sufficient to reconstitute reaggregation, are consistent with a two-site model. One site, the contact site, mediates the Ca^{2+}-dependent cell contact by the homophilic association of two identical contact sites on different molecules each associated with a different cell (Noll *et al.*, 1985). The other site, the butanol-sensitive cell binding site, in contrast to the contact site, is not genus specific because reconstitution is equally efficient within and across genera (Noll *et al.*, 1981). This lack of specificity implies that both the cell binding site of the complex and its receptor on the cell surface are highly conserved structures.

Since non-cytolytic extraction with butanol appears to remove only external membrane proteins (LeGrue, 1985), the complex must be bound to a transmembane protein similar to the receptors for other multifunctional external membrane proteins such as fibronectin, laminin, collagen and the related adherons (Schubert and LaCorbiere, 1985). The receptors for most of these external membrane proteins as well as many viruses share a highly conserved domain that recognizes the tripeptide Arg-Gly-Asp (Ruoslahti and Pierschbacher, 1986).

Experiments with monoclonal antibodies have shown that the 22S particles from blastula embryos were heterogeneous both with respect to structure and position. Thus, at least four classes of mAbs recognizing the 22S complex have been detected that bind to cell surface structures of the blastula in different patterns consistent with a code that specifies the position of a cell in the embryo by a unique combination of subunits in the 22S complex or toposome (Noll *et al.*, 1985).

For the isolation of morphogenetic cell adhesion molecules it was crucial to develop an assay that measures morphogenesis. Hence, previous cell interaction assays that rely on one parameter unrelated to morphogenesis such as the strength of cell interaction (Umbreit and Roseman, 1975; McClay *et al.*, 1981) or the rate at which single cells disappear into aggregates (Roth, 1968; Walther *et al.*, 1973; Brackenbury *et al.*, 1977) did not seem suitable for our purpose. Moreover, some of these assays appeared not to be practical for screening many samples containing small quantitites of active material as they involved shaking of relatively large volumes and number of cells (Moscona, 1961; Giudice, 1962; Timourian *et al.*, 1973; McClay and Hausman, 1975; McClay *et al.*, 1977; Urushihara *et al.*, 1979). By contrast, our assay in stationary microtiter wells requires small volumes and few cells and allows the simultaneous monitoring of many parameters, including quantitative estimates of the fraction of cells in aggregates and the average aggregate size as a function of time. Particularly important for our purpose, however, is an assessment of the quality of reaggregation and morphogenesis by direct and undisturbed microscopic inspection made possible by performing the assay in microtiter wells. Since it was introduced (Noll *et al.*, 1979), similar cell aggregation techniques using microtiter plates have been used by other investigators in a variety of applications (Hyafil *et al.*, 1980; Watanabe *et al.*, 1982; Shirayoshi *et al.*, 1983; Damsky *et al.*, 1983; McCarthy and Spiegel, 1983).

In the sea urchin, morphogenetic reaggregation occurs in several stages (Giudice, 1962). In the first few minutes, cells form small clumps by pseudopodial movements on the surface of the well bottom. Physiological cell contact in the initial clumping is recognized by the disappearance or blurring of the sharp cell boundaries as the surfaces of adjacent cells merge over large contact areas with concomitant loss of their rounded morphology. The clumps continue to grow until no aggregation competent

cells remain single in the vicinity of the aggregate. At this point, ~5–7 h after the start, a smooth epithelium with vigorously beating cilia is formed and the embryoids break free from the substratum to move vigorously, often in a spinning motion through the wells. The rate at which cells aggregate and the final size of the aggregates is determined by the initial cell density; $\sim 10^5$ cells is optimal for 100 μl of sea water in a standard well. Sometimes aggregates formed in the first hour stop morphogenesis and disintegrate later into a pile of rounded single cells.

Giudice (1965) has shown that this secondary dissociation was associated with an inhibition of metabolic activities, including protein and DNA synthesis as well as respiration, required for the transition from the lattice stage to the formation of an epithelial border. We suggest that this transition is dependent on a signal triggered by the interaction of contact sites and transmitted to the cytoplasmic domain of the transmembrane receptor. As a result, a cascade of secondary events is unleashed similar to those set in motion when growth factors and certain hormones, such as insulin, activate their receptors (Ullrich *et al.*, 1984, 1985).

Formation of an epithelial sheet is our most important criterion for morphogenetically significant reaggregation; we do not consider reaggregation to be valid if the cells fail to form aggregates with a smooth surface and if the individual rounded cell contours are still visible, as in the patterns published by McCarthy and Spiegel (1983). We emphasize this point because good positive controls are essential as a basis on which to judge inhibition.

In agreement with studies on cell–substratum adhesion with hamster kidney fibroblastic cells (Wylie *et al.*, 1979) and with compaction studies using mouse embryonal carcinoma cells (Ducibella, 1980), we find divalent antibodies (antiserum or IgG) equally well suited to produce the specific inhibitory effects seen previously only with monovalent Fab. Specificity and quantitation are achieved by titrating serial dilutions of the test fractions against a constant reaggregation inhibiting level of antibody added as IgG, Fab or antiserum. Although the patterns of inhibition differ for Fab and IgG or serum, the endpoint is unambiguously recognized in both cases by the transition from a pattern of inhibition as seen in the presence of antibodies alone to a pattern of morphogenetic reaggregation similar to the controls lacking antibodies. Unspecific agglutination resulting from divalent IgG directed against cell surface components not involved in cell adhesion was no problem because such agglutinations were transient and followed by normal reaggregation and development. Moreover, in testing dozens of antisera and their monovalent Fab, we have always found a strict congruence of their inhibitory titers. Finally, the demonstration that only the purified 22S complex and no other fractions tested could reverse the blocking effect of antiserum against whole membranes seems to rule out any non-specific effects. Characteristic for contact site-specific sera was an agglutination pattern in which the contours of the individual cells remained distinct instead of disappearing as in morphogenetic reaggregation. Only at low concentrations of the blocking serum was there a slow recovery from inhibition resulting in reaggregation.

The validity of the assay described in this paper is best illustrated by the fact that it has led to the identification of the toposomes, a new class of morphogenetically active cell adhesion particles that we have shown here to be the sole structure responsible for the adhesive integrity of the sea urchin blastula.

Because of its abundance in unfertilized eggs and packaging into 'yolk' platelets together with major yolk lipoproteins, the 22S complex has been regarded by some investigators as a vitellogenin (Shyu *et al.*, 1986). However, apart from this similarity in origin, all other characteristics of the intracellular 22S complex make it much more similar to a precursor and storage form of a cell surface protein. These characteristics include the glycoprotein composition, binding to lectins, presence in males as well as females and the developmentally regulated processing.

After completion of these studies, it was shown that the yolk granules can be separated physically into a high and low density class (Armant *et al.*, 1986). The existence of these two types of platelets had been previously observed in thin sections by electron microscopy (Gibbons *et al.*, 1969; Anderson, 1970; Wessel and McClay, 1985). It seems significant that the 22S complex was found to be exclusively associated with the low density granules (Armant *et al.*, 1986; Gratwohl *et al.*, in preparation) which are the main source of glycoproteins but do not contribute significantly to the total lipid content of the embryo (Armant *et al.*, 1986). Moreover, our toposomes (Gratwohl *et al.*, in preparation) as well as two germ layer-specific cell surface glycoproteins, whose SDS–gel patterns resemble those of toposomes (Wessel and McClay, 1985), are detected in the low density granules as well as on the cell surface upon staining with gold-labeled antibodies. Together these findings argue strongly that the low density granules are storage vesicles for membrane glycoproteins whose classification as 'yolk' or 'vitellogenin' must be questioned unless these terms connote any relatively abundant stored proteins regardless of structure and function. Storage of prospective cell surface components in the egg make sense, since development from egg to pluteus consists largely of the rapid conversion of cytoplasm into membranes and extracellular matrix. The recent cloning of the toposomes should further clarify these relationships (Shyu *et al.*, 1986; M.Noll *et al.*, unpublished results).

Materials and methods

The results described in this paper have been obtained with the five species enumerated below although only the three genera *Paracentrotus, Arbacia* and *Tripneustes* have been selected as representative examples. While the techniques of dissociation vary somewhat depending on how strongly the cells of a particular genus adhere to each other, the examples given are expected to cover the entire spectrum of cell affinities. The patterns of reaggregation and of inhibition by specific antibodies, on the other hand, have always been found to be the same regardless of genus.

Dissociation of embryos into single cells

Blastulae are harvested after hatching by centrifugation in a 50 ml graduated tissue culture centrifuge tube, washed once with millipore-filtered sea water (MFSW) and 0.5–1.0 ml of packed embryos resuspended in 45 ml of dissociation medium (DM). The DM varies according to genus, in order of decreasing ease of dissociation: Ca^{2+}, Mg^{2+}-free sea water (CMFSW) containing 2 mM EDTA or 1 mM EGTA for *P. lividus*, CMFSW containing 10 mM EDTA for *A. lixula, A. punctulata* and *Lytechinus variegatus*, 1 M glycine, 2 mM EDTA (Kane, 1973) for *T. gratilla*. For genera not mentioned here, the mildest conditions of dissociation should be tested first, progressing to stronger conditions as needed. The embryos are spun down, resuspended in 5 ml DM and inspected under the microscope. If dissociation is extensive (≥50%), it can usually be completed by repeated pipetting with a blunt-ended Pasteur pipette. If dissociation is marginal, a second centrifugation through 45 ml followed by resuspension in 5 ml of DM will normally bring it to completion. The tube is then filled with 10 mM Tris–MFSW pH 8.3 containing streptomycin and penicillin or gentamycin (reaggregation medium RM) and the cells spun into a pellet. After an additional wash with the same medium, the pellet is resuspended in a 40-fold volume of RM, corresponding to $\sim 10^5$ cells/5 μl. Inspection under the microscope should show small clumps of reaggregating cells. If reaggregation is poor, it can often be improved by an additional wash with RM to restore the physiological Ca^{2+}, Mg^{2+} levels. The cells are prevented from extensive reaggregation by frequent up and down pipetting with a blunt-ended Pasteur pipette. Pipettes with narrow tips should be avoided as they cause cell breakage by shear. For the same reason, centrifugal forces should be kept below 200 *g*.

Reaggregation assay

Standard assay. Microtiter plates with transparent flat bottoms are recommended. Plates that have not been treated to promote cell adherence are preferred because the treated plates commonly used for cell culturing prevent the detachment of the reaggregates and hence development to free swimming blastulae. To the first row of the 96-well microtiter plate (Linbro No. 76-203-05, Flow Laboratories) containig 90 μl of RM, we add in duplicate 5, 10 and 15 μl of cell suspension and allow the cells to settle to the bottom for 5–10 min. The wells are then inspected under the microscope and the optimal cell input (~ 10^5 cells/well) determined from the patterns produced. Optimal reaggregation and development is obtained when the cells do not form a monolayer but are well separated by about one cell diameter on the average (Noll *et al.*, 1981). The optimal volume of stock cell suspension is then selected by comparison of the patterns, if necessary by interpolation. The volume so chosen e.g. 7 μl, is then added quickly to each well with a disposable tip micropipetter. The cells are always added last and in the direction of increasing concentration of the test substance present in the wells to minimize the effect of carry over when using the same tip for each dilution series. After each addition, the well is stirred with the pipette tip to ensure uniform distribution of the cells.

The microtiter plates are always prepared to contain all the components to be tested in 90 μl of RM before we start the harvesting and dissociation of the embryos in order to reduce the time between dissociation and addition of the cells to the wells to an absolute minimum. This is important because reaggregation starts quickly after the cells are brought back to physiological Ca^{2+} and Mg^{2+} concentrations. Rapid restoration of these ions is essential, since otherwise the cells lose their capacity for reaggregation and development.

Cell density. To evaluate the influence of cell density, we used the techniques described above, except that the cell density was varied as indicated.

Evaluation. The plates were inspected under the inverted microscope at hourly intervals until 7 h and again after 16–24 h and the results recorded by microphotography.

Serum, IgG and Fab. Antisera were collected from individual rabbits immunized with butanol extracts from purified membranes of blastula embryos of *P. lividus* (rabbit No. 1223 B), *A. lixula* (1221 B) or *T. gratilla* (215). Sera were obtained from 12 rabbits for each genus with reaggregation inhibiting titers ranging from >10 to 0.25 μl. For our experiments we selected sera with inhibitory endpoints of ≤2.5 μl. Antibodies from rabbits 1223B and 1221B have been used in our earlier work (Noll *et al.*, 1981) which also describes the isolation of IgG from serum by affinity chromatography on Sepharose–protein A. IgG was digested with mercuripapain (Sigma) at a concentration of 2–5 μg/mg IgG at 37°C for 4–6 h and separated from F_c on a protein A affinity column (Noll *et al.*, 1981). The purity of both IgG and Fab were monitored on SDS gels and by high pressure gel permeation chromatography on a G4000SW 7.5 × 300 mm column. Fab, IgG and serum were dialyzed against MFSW before addition to the assay mixtures. In some experiments serum was added without dialysis after tests had shown that up to 40 μl serum per 100 μl assay mixture had no deleterious effect upon reaggregation.

Membranes, butanol extract and 22S complex. These components were prepared as described previously (Noll *et al.*, 1985). For large-scale preparation of 22S complex, butanol extract from purified membanes (Noll *et al.*, 1979) or cytosol was passed in MFSW through a S-400 Sephacryl column (80 cm × 2.5 cm). The cytosol was obtained by collecting the supernatant after centrifugation of the crude embryo homogenate at 10 000 r.p.m. for 20 min at 4°C in a Sorvall SS-34 rotor. The 22S complex emerges as a sharp peak after two broader peaks in a pattern similar to that obtained from Bio-gel A-50 m by Il *et al.* (1978) with supernatant from yolk homogenates. In agreement with their findings, the two preceding peaks consist of low density yolk lipoproteins that fail to sediment in a 5% (top) isokinetic sucrose gradient (Noll, 1967). Rechromatography of peak 3 on Sephacryl S-400 or sedimentation through a sucrose gradient yield essentially pure 22S complex as assessed by SDS-PAGE (Il *et al.*, 1978; Kari and Rottmann, 1985; Noll *et al.*, 1985).

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